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Threats from wildlife trade: The importance of genetic data in safeguarding the endangered Four-eyed Turtle (*Sacalia quadriocellata*)

Minh Duc Le[1,2,3*], Timothy E.M. McCormack[4], Ha Van Hoang[4], Ha Thuy Duong[5], Truong Quang Nguyen[6,7], Thomas Ziegler[8,9], Hanh Duc Nguyen[10], Hanh Thi Ngo[2,5*]

1 *Department of Environmental Ecology, Faculty of Environmental Sciences, University of Science, Vietnam National University, Hanoi, 334 Nguyen Trai Road, Hanoi, Vietnam* **2** *Central Institute for Natural Resources and Environmental Studies, Vietnam National University, Hanoi, 19 Le Thanh Tong, Hanoi, Vietnam* **3** *Department of Herpetology, American Museum of Natural History, Central Park West at 79th Street, New York, USA* **4** *Asian Turtle Program, Room 1806 CT1, C14 Bac Ha Building, To Huu Street, Nam Tu Liem District, Hanoi, Vietnam* **5** *Department of Genetics, Faculty of Biology, University of Science, Vietnam National University, Hanoi, 334 Nguyen Trai Road, Hanoi, Vietnam* **6** *Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Hanoi, Vietnam* **7** *Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay, Hanoi, Vietnam* **8** *AG Zoologischer Garten Köln, Riehler Strasse 173, D-50735 Köln, Germany* **9** *Institute of Zoology, University of Cologne, Zülpicher Strasse 47b, D-50674 Köln, Germany* **10** *Hanoi Procuratorate University, Duong Noi, Ha Dong, Hanoi, Vitenam*

Corresponding author: Minh Duc Le (le.duc.minh@hus.edu.vn)

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Abstract

Wildlife trade has been considered one of the largest threats to biodiversity in Southeast Asia. Many vertebrates, such as pangolins, elephants and turtles have been heavily hunted as a result of high demand from emerging markets in China and other countries in the region. In Vietnam, over-exploitation of turtles over several decades to supply the international trade has extirpated numerous populations and driven several species to the brink of extinction. To reverse this trend, conservation measures, such as re-introduction

* Contributed equally as the first author.

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of confiscated or captive-bred animals to their native habitats, should be implemented to recover severely declined local populations. For species with a complex phylogeographic structure, however, it is crucial to understand geographic patterns of genetically-distinct populations to avoid releasing animals of unknown origin to wrong localities. In this study, we investigate the phylogeographic pattern of the Four-eyed Turtle (*Sacalia quadriocellata*), a widely traded species, which occurs in southern China, northern and central Laos and much of Vietnam, using samples with known localities and those collected from the local trade. Our range-wide phylogenetic and network study, based on the complete mitochondrial cytochrome *b* gene, recovered at least three major clades and seven subclades within the species range. Amongst these, two subclades, one from northern Annamites, Vietnam and the other from north-eastern Laos, are newly discovered. The fine scale phylogeographic analysis helped us to assign misidentified sequences from GenBank and those from confiscated animals with unknown origin to well-defined geographic populations. The results highlight the importance of incorporating samples collected from the local trade and the wild in genetic analyses to support both *ex-situ* and *in-situ* conservation programmes of highly-threatened species in accordance with the IUCN's One Plan Approach.

Keywords

conservation, cytochrome *b*, Lao PDR, *Sacalia quadriocellata*, Vietnam, wildlife trade

Introduction

Unsustainable exploitation of wildlife and their products has been recognised amongst the most serious threats to biodiversity conservation in Southeast Asia and to the survival of many globally-threatened wildlife species (Nijman 2010; Bennett 2011; Auliya et al. 2016; Krishnasamy and Zavagli 2020). According to Schneider (2008), the value from illicit trafficking of wildlife species and their products in the world is only ranked behind illegal trade in weapons and drugs. Experts estimate illegal global trade of wildlife to be worth at least $5 billion and possibly up to $20 billion per year (Wyler and Shikh 2013; Lawson and Vines 2014). This number can be converted into millions of wildlife individuals, particularly large mammals, birds and reptiles, which are poached and traded across national borders annually in the context of increasing demands for consumption and use.

In Vietnam, wildlife trade activities started to grow exponentially at the end of the 1980s when Vietnam opened its market to international trade, especially to China. In recent years, trade activities have still occurred widely and are likely to expand. The growth of wildlife trade has been illustrated through the number of export permits issued by Vietnam's CITES Office annually and documented illegal activities (WCS 2012; Janssen and Indenbaum 2019). In the global wildlife trade and consumption network, Vietnam is considered to play many roles, including three major ones: exporting, consuming and trafficking (Lawson and Vines 2014; Milliken 2014; Challender et al. 2015; Tran et al. 2016; Krishnasamy and Zavagli 2020).

Turtles have been collected and exported from Vietnam to China in large quantities since the late 1980s (Le and Broad 1995; Hendrie 2000; Le 2007). The trade has

continued to flourish in recent years with massive volumes recorded. For example, between 2005 and 2010, the Wildlife Crime Unit of the Education for Nature, Vietnam, an NGO focusing on countering wildlife trafficking, reported 163 smuggling cases of freshwater turtles and tortoises in the country, accounting for more than 25 tonnes or approximately 30,000 individuals (ENV 2010). As a result of a long period of overexploitation, many turtle populations in Vietnam have seriously declined and some species are considered virtually extinct or functionally extinct in the wild, such as the Vietnamese Three-striped Box Turtle (*Cuora cyclornata*), the Vietnamese Pond Turtle (*Mauremys annamensis*) and the Swinhoe's Softshell Turtle (*Rafetus swinhoei*).

Recently, the trade has taken advantage of the popularity of social networks in the country to sell turtles on these platforms. The number of turtles advertised for sale on Facebook, Myspace and Twitter has sharply escalated (Tran et al. 2016; Pham et al. 2019). According to a survey from March to May 2015, 346 turtle individuals of 15 different species were displayed on online markets nationally (Tran et al. 2016). An investigation into illegal trade activities on Vietnamese Facebook groups between 2013 and 2018 documented 481 turtle advertisements, involving almost 6000 individuals of 53 species and 12 families. Astoundingly, only around 42% or 22 species of those were native to Vietnam (Pham et al. 2019).

Vietnam is home to 29 species of freshwater turtles and tortoises (Turtle Taxonomy Working Group 2017) and as many as 24 of them are listed as Vulnerable or higher in the IUCN Red List (2020) (Fig. 1). One highly threatened, but largely neglected species is the Four-eyed Turtle (*Sacalia quadriocellata*), which occurs in China, Vietnam and Laos (Zhao 1998; Stuart et al. 2001; Shi et al. 2008; Suzuki et al. 2015; Turtle Taxonomy Working Group 2017). It is listed as Endangered by the IUCN Red List (2020) and included in Appendix II of the Convention on

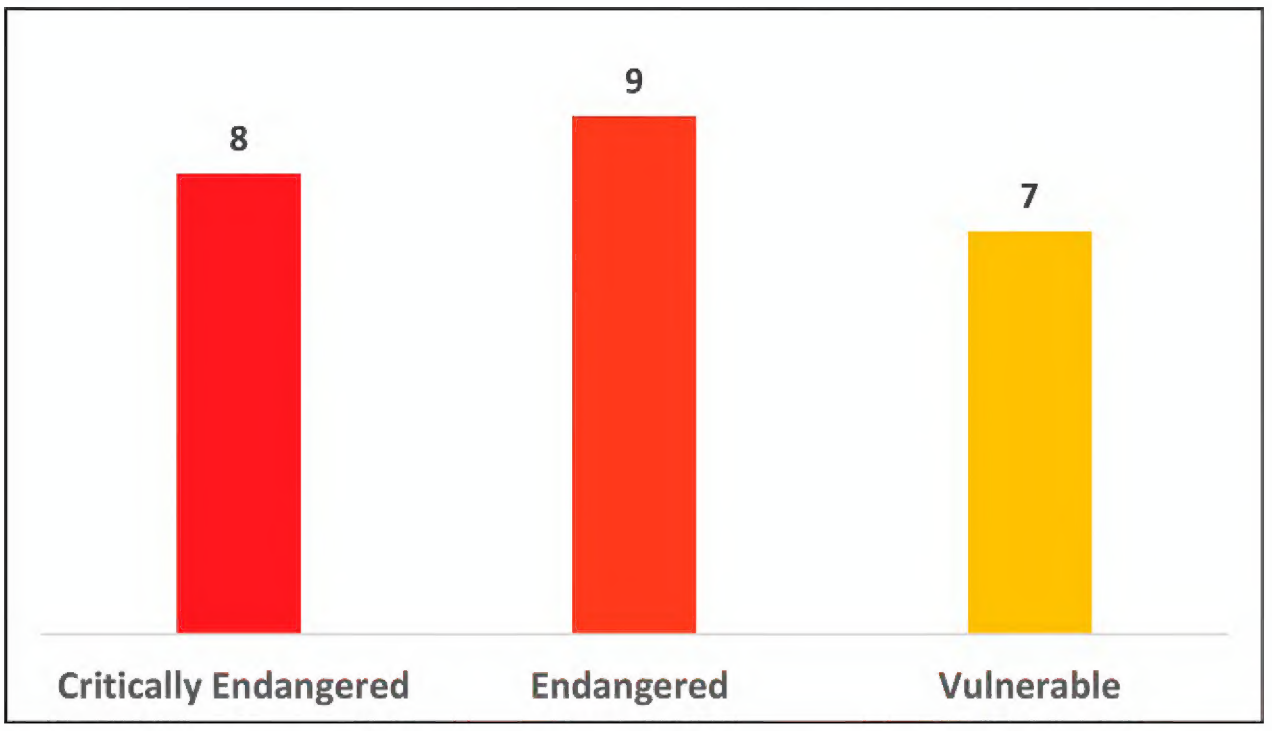

Figure 1. Conservation status of freshwater turtles and tortoises in Vietnam (IUCN Red List 2020).

International Trade in Endangered Species of Wild Fauna and Flora (CITES). The population of the species has been reportedly declining in China, Laos and Vietnam due to a high level of poaching and habitat loss (He et al. 2010; Suzuki et al. 2015; Tran et al. 2016), but no assessment is currently available for the species in any part of its range (IUCN Red List 2020). Over the last three years, 25 individuals of *Sacalia quadriocellata* were recorded in the trade in Vietnam by the Institute of Ecology and Biological Resources (Hanoi), comprising 22 turtles seized in Nghe An Province and three confiscated in Kon Tum Province.

The species has been shown to contain a high level of diversity with a number of genetically-distinct and geographically-isolated populations identified in a previous study (Shi et al. 2008). To our knowledge, these well-differentiated populations have not been recognised as separate conservation units in any conservation programme for the species, except for the population from Hainan of China, which has been proposed as a distinct species, *Sacalia insulensis* (Lin et al. 2018). This situation could lead to serious problems down the line, including mixing independently evolving lineages in conservation breeding facilities and releasing non-native animals to natural habitats, resulting in genetic pollution of local genetic pools. To further investigate the cryptic diversity and distribution of the poorly-studied species and clarify patterns of genetic differentiation of the species in Vietnam, we conducted both field and trade surveys across the range of the species with a focus in Vietnam and Laos. We sequenced the complete mitochondrial cytochrome *b* from collected DNA samples from wild populations and local trades and recovered their phylogenetic and network relationships using published and newly-generated sequences. Based on the results, we recommend options for conservation management of the species.

Materials and methods

Interview survey

A total of 2,758 interviews were conducted in 30 Provinces from northern to south central Vietnam from 2010–2018 with 79 turtle individuals observed. A short survey was also undertaken in Nam Xam Biodiversity Conservation Area, Xam Tai District, Houaphan Province in Laos from 25 May to 12 June 2015 with 118 interviews completed and 27 live specimens recorded. We conducted interviews using a semi-structured survey technique (Creswell and Poth 2018) with a focus on local people living around protected areas, as well as professional hunters and traders. Information on turtle origin, measurements and photos of individuals encountered during those interview surveys were recorded. Tissue samples for molecular analysis were collected from either tail tips or oral swabs and stored in 70% alcohol when possible. Geographic coordinates and elevation were recorded using a Garmin GPSMAP GPS 60CSx receiver in datum WGS 1984.

Field survey

Field surveys were conducted in three areas: 1) from 24 May to 3 June 2010 in Quang Nam Province at Cha Val Commune, Song Thanh Nature Reserve; 2) from 8 to 17 August 2008 at Khe Hua and Khe Phung Cam in Pu Huong Nature Reserve; and 3) from 9 to 19 June 2010 at Ban Bung – Khe Ca area in Pu Mat National Park, Nghe An Province, in north-central Vietnam (Fig. 2). The survey areas were characterised by evergreen forest and 30–50 degree slopes. Elevation ranged from 330 to 1000 m. We combined diurnal and nocturnal surveys, focusing on suitable habitats. Fifteen non-lethal aquatic turtle traps with chicken guts and rotten meat used as baits were also deployed to capture the Four-eyed Turtle in Khe Hua and Khe Phung Cam (Hua and Phung Cam Streams) in Pu Huong Nature Reserve and Khe Ca (Ca Stream) in Pu Mat National Park. Traps were checked every morning to change baits. The search in forests started at 6 am and ended around 5 pm. Measurements, information on habitat and ecology, tissue samples and photos were taken from turtles captured at the sites. Geographic coordinates and elevation were obtained using a Garmin GPSMAP GPS 60CSx receiver and recorded in datum WGS 1984.

Taxonomic sampling

In total, 20 new samples of *Sacalia quadriocellata* were incorporated in the analysis, including five wild collected samples with three from Pu Mat National Park and one each from Pu Huong Nature Reserve, Nghe An Province and Song Thanh Nature Reserve, Quang Nam Province. Another 13 were collected from local trade where the turtles were kept in local households from the area bordering with China, Cao Bang Province to the southern-most known localities of the species, Khanh Hoa Province in Vietnam and Houaphan Province, north-eastern Lao PDR. Two other samples were taken from confiscated animals in Pleiku City, Gia Lai Province in the Central Highlands, Vietnam (Table 1, Fig. 2). In addition, we obtained 34 sequences of the mitochondrial cytochrome *b* from GenBank for *Sacalia quadriocellata* and *S. bealei* and three others for outgroup taxa, *Cuora trifasciata*, *Cyclemys dentata* and *Mauremys annamensis*. Amongst these, 29 originated from Shi et al. (2008).

Molecular data

Total genomic DNA was extracted using the Dneasy Blood and Tissue Kit (Qiagen – Hilden, Germany) following the manufacturer's instructions for animal tissue. The genomic extraction was checked by electrophoresis. A negative control was used for every extraction.

We amplified the complete mitochondrial cytochrome *b* for all samples using HotStar Taq Mastermix (Qiagen – Hilden, Germany) and Dream Taq PCR Mastermix (Thermo

Table 1. GenBank accession numbers and associated voucher/laboratory numbers of ingroup taxa used in this study.

Species names	GenBank N	Voucher/Lab number	Reference	Origin	Locality
Sacalia bealei (5)	EU910982	HNUTSB0	Shi et al. 2008	Pet trade	–
Sacalia bealei (6)	EU910983	HNUTSB28	Shi et al. 2008	Pet trade	–
Sacalia bealei (7)	EU910984	HNUTSB19	Shi et al. 2008	Pet trade	–
Sacalia bealei (8)	AJ519501	MTD 41583	Barth et al. 2004	–	–
Sacalia bealei (9)	EU910981	HNUTSB25	Shi et al. 2008	Pet trade	–
Sacalia bealei (10)	EU910992	MVZ257748	Shi et al. 2008	Wild collected	Hong Kong, China
Sacalia bealei (11)	AY434585	HBS38403	Spinks et al. 2004	Pet trade	–
Sacalia bealei	GU183364	–	Nie and Jang 2016	–	–
Sacalia bealei	HQ442416	ANUM26080081	Xia et al. 2011	–	–
Sacalia bealei	NC016691	–	Nie and Jang 2012	–	–
*Sacalia bealei**	EF088646	–	Nie and Song 2016	–	–
Sacalia quadriocellata (12)	FJ211058	MVZ 258023	Shi et al. 2008	Wild collected	Quang Nam, Vietnam
Sacalia quadriocellata (13)	EU910995	FMNH 256542	Shi et al. 2008	Wild collected	Khammouane, Laos
Sacalia quadriocellata (14)	EU910994	FMNH 256543	Shi et al. 2008	Wild collected	Khammouane, Laos
Sacalia quadriocellata (15)	FJ211059	ZFMK 81536	Shi et al. 2008	Wild collected	Ha Tinh, Vietnam
Sacalia quadriocellata (16)	FJ211060	ZFMK 81535	Shi et al. 2008	Wild collected	Ha Tinh, Vietnam
Sacalia quadriocellata (17)	EU910974	HNU TSQ11	Shi et al. 2008	Pet trade –	
Sacalia quadriocellata (18)	AJ564465	MTD 42442	Barth et al. 2004	Pet trade	–
Sacalia quadriocellata (19)	EU910973	HNU TSQ8	Shi et al. 2008	Pet trade	–
Sacalia quadriocellata (20)	EU910993	ROM 28458	Shi et al. 2008	Local trade	Tuyen Quang, Vietnam
Sacalia quadriocellata (21)	EU910990	–	Shi et al. 2008	Pet trade	–
Sacalia quadriocellata (22)**	AY434614	HBS 38436	Spinks et al. 2004	Pet trade	–
Sacalia quadriocellata (23)	EU910988	HNU TSQ4	Shi et al. 2008	Pet trade	–
Sacalia quadriocellata (24)	EU910987	HNU TSQ3	Shi et al. 2008	Pet trade	–
Sacalia quadriocellata (25)	EU910991	MVZ 257747	Shi et al. 2008	Wild collected	Guangdong, China
Sacalia quadriocellata (28)	EU910985	HNU TSQ281	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (29)	EU910975	HNU TSQ224	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (30)	EU910989	HNU TSQ61	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (31)	EU910978	HNU TSQ264	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (32)	EU910986	R0520	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (33)	EU910980	HNU TSQ273	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (34)	EU910976	HNU TSQ231	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (35)	EU911001	MVZ 230485	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (36)	EU911000	MVZ 230484	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (37)	EU910977	HNU TSQ239	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata (38)	EU910979	HNU TSQ284	Shi et al. 2008	Wild collected	Hainan, China
Sacalia quadriocellata	GU320209	–	Nie and Jiang 2016	–	–
Sacalia quadriocellata	MT845096	SAC 7	This study	Local trade	Ha Tinh, Vietnam
Sacalia quadriocellata	MT845097	SAC 9	This study	Local trade	Ha Tinh, Vietnam
Sacalia quadriocellata	MT845098	SAC 10	This study	Local trade	Ha Tinh, Vietnam
Sacalia quadriocellata	MT845099	SAC 11	This study	Local trade	Quang Ninh, Vietnam
Sacalia quadriocellata	MT845100	SAC 12	This study	Local trade	Quang Ninh, Vietnam
Sacalia quadriocellata	MT845101	SAC 13	This study	Local trade	Quang Ninh, Vietnam
Sacalia quadriocellata	MT845102	SAC 15	This study	Local trade	Thua Thien Hue, Vietnam
Sacalia quadriocellata	MT845103	SAC 16	This study	Local trade	Houaphan, Laos
Sacalia quadriocellata	MT845104	SAC 17	This study	Local trade	Houaphan, Laos
Sacalia quadriocellata	MT845105	SAC 18	This study	Local trade	Houaphan, Laos
Sacalia quadriocellata	MT845106	SAC 19	This study	Confiscated	–
Sacalia quadriocellata	MT845107	SAC 20	This study	Confiscated	–
Sacalia quadriocellata	MT845108	SAC 21	This study	Wild collected	Nghe An, Vietnam
Sacalia quadriocellata	MT845109	SAC 22	This study	Wild collected	Nghe An, Vietnam
Sacalia quadriocellata	MT845110	SAC 23	This study	Wild collected	Nghe An, Vietnam
Sacalia quadriocellata	MT845111	SAC 24	This study	Wild collected	Nghe An, Vietnam
Sacalia quadriocellata	MT845112	SAC 25	This study	Local trade	Cao Bang, Vietnam

Species names	GenBank N	Voucher/Lab number	Reference	Origin	Locality
Sacalia quadriocellata	MT845113	SAC 26	This study	Local trade	Khanh Hoa, Vietnam
Sacalia quadriocellata	MT845114	SAC 27	This study	Local trade	Binh Dinh, Vietnam
Sacalia quadriocellata	MT845115	SAC 28	This study	Wild collected	Quang Nam, Vietnam

* This sequence record is labelled as *S. quadriocellata* on GenBank; ** This sequence record is labelled as *S. pseudocellata* on GenBank, a hybrid species, but still considered to be *S. quadriocellata* in Shi et al. 2008. Numbers in parentheses are marked following Shi et al. (2008).

Fisher Scientific – Vilnius, Lithuania). The standard PCR conditions were 95 °C for 15 min with HotStar Taq Mastermix (Qiagen – Hilden, Germany) or 95 °C for 5 min with Dream Taq PCR Mastermix (Thermo Fisher Scientific – Vilnius, Lithuania), 35 cycles at 95 °C for 30 s, 45 °C for 45 s, 72 °C for 60 s; a final elongation at 72 °C for 6 min. The PCR volume consisted of 2 µl of each primer at 10 pmol/µl, 5 µl water, 10 µl of Mastermix and 1–4 µl DNA template depending on the quantity of DNA. All primers used for this study are listed in Table 2. A negative and positive control was used for every PCR reaction. To confirm if the correct size was amplified, 5 µl of PCR product was run on a 1% agarose gel, 1X TBE buffer, stain with 2 pg/µl bromide and photographed under UV light. Successful PCR products were cleaned using Gene Jet PCR Purification Kit (Thermo Fisher Scientific – Vilnius, Lithuania) following manufacturer's instructions. Cleaned PCR products were sent to 1st Base (Malaysia) for sequencing.

Phylogenetic and network analyses. The sequences were edited using Sequencher v4.1.4 (Gene Codes Corp, Ann Arbor, MI, USA) then aligned using Bioedit v7.1.3 (Hall 1999) with default settings. Data were analysed using three phylogenetic methods, Maximum Parsimony (MP) as implemented in PAUP*4.0b10 (Swofford 2001), Bayesian Inference (BI) in MrBayes v3.2.7 (Ronquist et al. 2012) and Maximum Likelihood (ML) in IQ-TREE v1.6.8 (Nguyen et al. 2015), respectively. Intraspecific relationships amongst populations of *Sacalia quadriocellata* were also inferred using the NeighborNet algorithm (Bryant and Moulton 2004) using SplitsTree v4.14.2 (Huson and Bryant 2006). For MP analysis, heuristic analysis was conducted with 100 random taxon addition replicates using tree-bisection and reconnection (TBR) branch swapping algorithm, with no upper limit set for the maximum number of trees saved. All characters were equally weighted and unordered. For BI, we performed both single and multiple models by codon partitions to examine the robustness of the tree topology (Nylander et al. 2004; Brandley et al. 2005). Analyses were conducted with a random starting tree and run for 1×10^7 generations, four Markov chains (one cold, three heated) with default settings. Values of sample points were plotted against the number of generations to detect stationarity of the Markov chains. Trees generated prior to stationarity were removed from the final analyses using the burn-in function. Two independent analyses were performed simultaneously. The cut-off point for the burn-in function was set to 25 and 28 in the single- and multiple-model Bayesian analyses, respectively, as $-\ln L$ scores reached stationarity after 25,000 and 28,000 generations in both runs of the two analyses. The posterior probability (PP) values for all clades in the final majority rule consensus tree were provided (Fig. 3). Nodal support was also evaluated using Bootstrap replication (BP) as calculated in PAUP (1,000

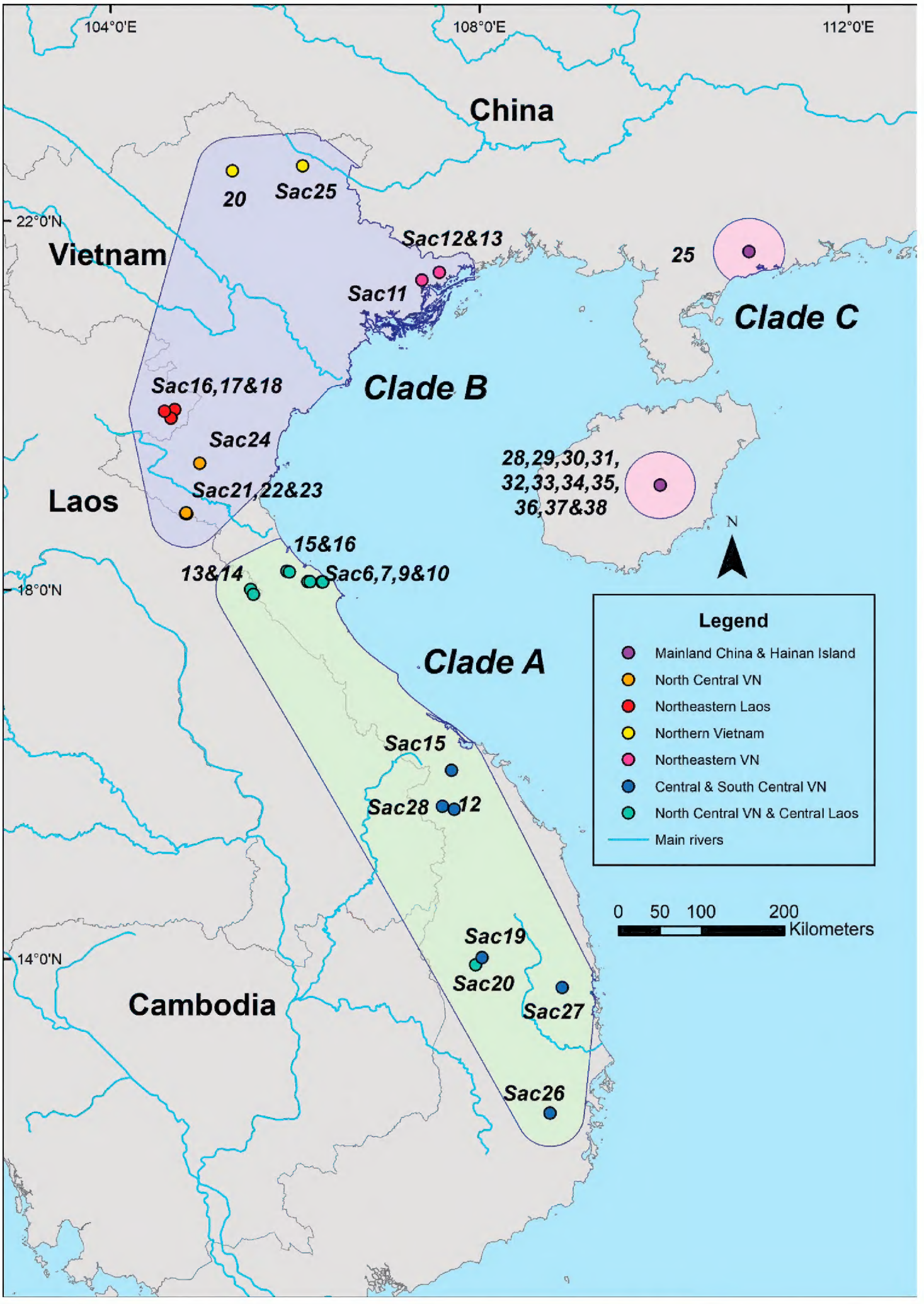

Figure 2. Samples of *Sacalia quadriocellata* collected from the field and local trade. Three delineated areas, A, B and C, represent distributions of three phylogeographic clades shown in Figs 3, 4. See Table 1 for more information of the samples.

Table 2. Primers used in this study.

Primer	Sequence (5' to 3')	Reference
CytbG (f)	AACCATCGTTGTWATCAACTAC	Spinks et al. (2004)
GLUDGE (f)	TGATCTTGAARAACCAYCGTTG	Palumbi et al. (1991)
CytbJSi (f)	GGATCAAACAACCCAACAGG	Spinks et al. (2004)
CytbJsr (r)	CCTGTTGGGTTGTTTGATCC	Spinks et al. (2004)
THR-8 (r)	GGTTTACAAGACCAATGCTT	Spinks et al. (2004)

(f): forward, (r): reverse

replications and 100 random taxon addition) and in SplitsTree (1000 replications) and employing ultrafast BP (10,000 replications) in IQ-TREE. We regarded BP value ≥ 70% and PP value ≥ 95% as strong support for a clade (Hillis and Bull 1993; Ronquist et al. 2012). For ML and BI analyses, we used the optimal model calculated by jModelTest v.2.1.10 (Darriba et al. 2012). The optimal model for nucleotide evolution was set to TVM+I for single model BI and ML analyses. For the Bayesian multiple model analysis, three selected models, TVMef+G, HKY+I and TrN+I, for three codon positions were assigned to these partitions in MrBayes using the command APPLYTO. Model parameters were inferred independently for each data partition using the UNLINK command. Uncorrected pairwise genetic divergences between different lineages of *Sacalia quadriocellata* were calculated in PAUP*4.0b10. The network analysis was performed in SplitsTree with the following settings: edge fitting as ordinary least squares, equal angle as chosen splits transformation, least squares to modify weights and four maximum dimensions as the filtering option. The generated split graph showed a visual representation of conflicting signals in the data by presenting them as a series of parallel edges. The programme computed the least squares fit (LSfit) between the pairwise distances from the graph and the distances from the matrix to produce a distance-based unrooted tree diagram by means of the neighbour-joining algorithm (Saitou and Nei 1987).

Results

We successfully sequenced the complete cytochrome *b* gene for 20 samples collected from eight Provinces in Vietnam, one Province in Lao PDR and two samples from confiscated animals. The final matrix consisted of 1143 aligned characters with no gap, of which 215 characters were parsimony informative and 822 were constant in the MP analysis. In total, 48 most parsimonious trees were found (Tree length = 504; Consistency index = 0.7; Retention index = 0.92). In the ML analysis, the log likelihood of the best tree found was -9838.332. The three analyses produced well-corroborated phylogenies, although the MP tree is less resolved, compared to those estimated by ML and BI. Most major nodes of the trees received high statistical support from at least two analyses (BP ≥ 70% and PP ≥ 95%) (Fig. 3).

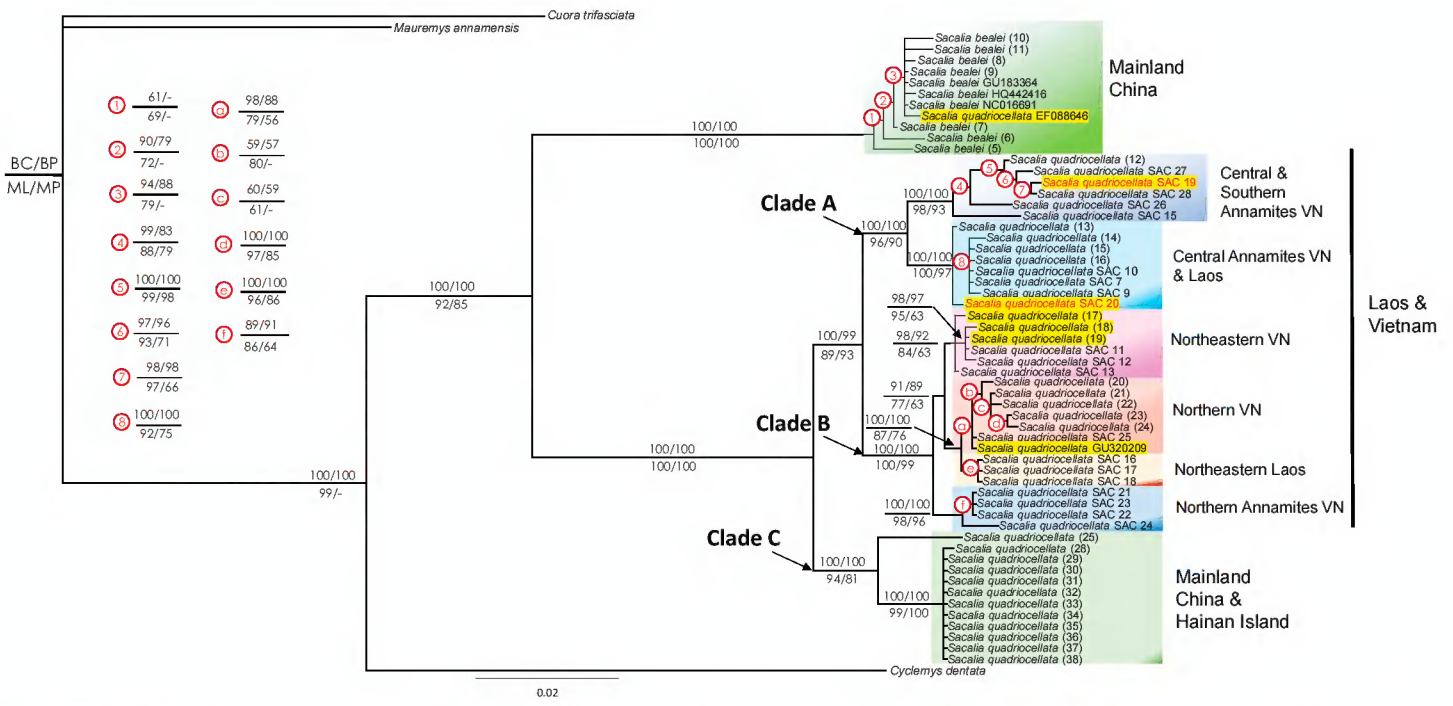

Figure 3. Bayesian cladogram, based on the complete mitochondrial cytochrome *b* gene. The numbers above and below branches are Bayesian posterior probability for single/multiple models and maximum likelihood/maximum parsimony bootstrap values, respectively (all in percentage). Highlighted samples in red and black letters denote those originating from confiscations and GenBank or the previous study with no reliable locality/identity, respectively.

Similar to results reported by Shi et al. (2008), our phylogenetic hypothesis strongly supports the monophyly of the genus *Sacalia* and its two species, *S. bealei* and *S. quadriocellata*. In addition, three main clades, Clade A, B and C, within *S. quadriocellata*, were well corroborated by all four analyses. Clade A included populations from north-central Vietnam and central Laos, including Ha Tinh and Quang Binh Provinces in Vietnam and Khammouane Province in Lao PDR, along with those from central Vietnam and south-central Vietnam, i.e. Thua Thien Hue, Quang Nam, Binh Dinh and Khanh Hoa Provinces (Figs 2, 3, Table 1). Both of the subclades within Clade A received strong statistical support from all four analyses. In this study, we expanded the north-central Vietnam and central Laos clade to include Ha Tinh Province (samples SAC 7, 9 and 10). One confiscated sample of unknown origin, SAC 20, was also grouped in this subclade.

The other subclade of Clade A contained a higher level of diversity than previously shown. The only sample incorporated in Shi et al. (2008), sample (12) from Song Thanh Nature Reserve, Quang Nam Province, was placed in a lineage (node 5) with another sample from the Reserve (SAC 28) and a sample from Binh Dinh Province (SAC 27) with high statistical support from all analyses. The second confiscated sample, SAC 19, also clustered in the group (Fig. 3). The populations from Thua Thien Hue Province (SAC 15), distributed north of Quang Nam and Binh Dinh Provinces and from Khanh Hoa Province (SAC 26), located in the southernmost locality of the species range, were divergent from the middle group (nodes 4 and 5) (Fig. 3).

In Clade B, four subclades were supported in our analyses instead of two in Shi et al. (2008). Two newly-discovered subclades included one from north-eastern Laos,

Houaphan Province and the other from north-central Vietnam, Nghe An Province (Table 1, Fig. 3). Both of the subclades received strong statistical support from all analyses. For two other subclades corroborated by Shi et al. (2008), our analyses were able to assign them to two separate biogeographic areas, the north-eastern Vietnam unit, consisting of samples from Quang Ninh Province (SAC 11, 12 and 13) and three samples of unknown origin (17, 18 and 19 from Shi et al. 2008) and the northern Vietnam unit, containing samples from Shi et al. (2008) with one from Tuyen Quang Province (sample 20) and a new sample (SAC 25) collected from neighbouring Cao Bang Province. A sample of unknown origin with GenBank accession number of GU320209 was also placed in this subclade (Figs 2, 3).

Clade C, basal to Clade A and Clade B, comprised one sample from mainland China, sample (25) and the rest from Hainan Island. This clade was the most divergent from those occurring in Vietnam and Laos with an average of approximately 3.5% in terms of genetic pairwise distance. The population from mainland China was around 2% differentiated from Hainan Island's population (Table 3). The network analysis generated the same results as those provided by phylogenetic estimations. In particular, three main clusters, Clade A, B and C, were also strongly supported by the Neighbor-Net algorithm and, amongst the clades, Clade A and B from Vietnam and Laos were more closely related to each other than to Clade C from mainland China and Hainan Island. In each main clade, subclades recovered by phylogenetic analyses, two in Clade A, four in Clade B and two in Clade C, were also corroborated, based on genetic distance in the network analysis (Fig. 4).

Discussion

The samples included in our study cover most of distribution localities of the Four-eyed Turtle reported in previous studies, for example, Shi et al. (2008) and Turtle Taxonomic Working Group (2017). Only those from several localities from mainland China,

Table 3. Uncorrected ("p") distance matrix showing percentage pairwise genetic divergence (cytochrome *b*) between different subclades within *Sacalia quadriocellata*. The highest pairwise distance within clades is italicised and shown in parenthesis.

Subclade name	CSAVN	CAVNLA	NEAVN	NORVN	NEALA	NORAN	HAINI	MACHI
CSAVN	(*1.8*)							
CAVNLA	1.5–2.5	(*0.5*)						
NEAVN	2.5–3.5	2.0–2.7	(*0.3*)					
NORVN	2.5–3.4	2.1–2.9	0.4–1.0	(*0.4*)				
NEALA	2.5–3.3	2.3–2.7	0.4–0.7	0.3–0.6	(*0*)			
NORAN	2.5–3.7	2.2–2.9	0.6–1.1	0.6–1.3	0.8–1.1	(*0.5*)		
MACHI	3.3–3.9	3.4–4.1	3.3–3.6	3.2–3.6	3.5–3.7	3.2–3.8	(*0.1*)	
HAINI	3.6–4.0	3.6–3.9	3.7–4.0	3.7–4.0	3.9–4.0	3.6–3.9	2.0–2.1	(*0*)

CSAVN: Central & southern Annamites, Vietnam; CAVNLA: Central Annamites, Vietnam and Laos, NEAVN: North-eastern Vietnam; NORVN: Northern Vietnam; NEALA: North-eastern Laos; NORAN: Northern Annamites; MACHI: Mainland China; HAINI: Hainan Island.

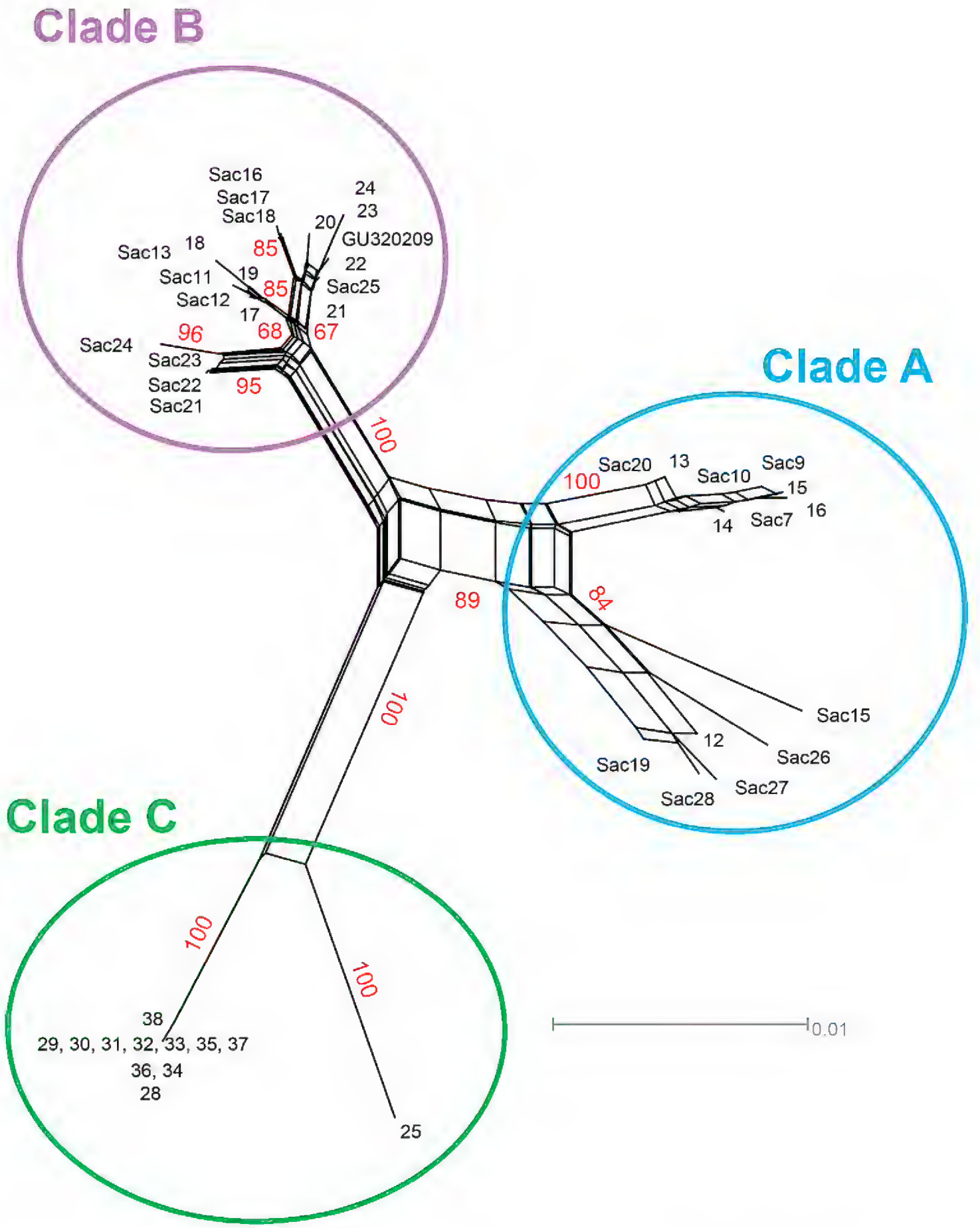

Figure 4. Split tree network, based on the complete mitochondrial cytochrome *b* gene. Red numbers at nodes are bootstrap values in percentage.

Hainan Island, northern Vietnam and possibly eastern Laos (Suzuki et al. 2015) were not included in the analysis. We also recorded the southernmost locality of the species in Khanh Hoa Province (Fig. 2), as, prior to this study, the southern end of the distribution of the Four-eyed Turtle was deemed to be in Binh Dinh Province (Turtle Taxonomic Working Group 2017). This population is also approximately 1.0–1.6% genetically

divergent from others distributed in central and south-central Vietnam. In general, the species shows substantial genetic diversity throughout its range and natural boundaries for each clade and subclade seem to follow river systems and mountain ranges.

The largely aquatic species exhibits a finer partitioned phylogeographic pattern compared to other species of turtles in the region (Stuart and Parham 2004; Blanck et al. 2006; Gong et al. 2009; Stuckas and Fritz 2011). The number of lineages recovered and their distribution in this study suggests the populations tend to be more isolated by natural boundaries compared to other reptiles and amphibians as demonstrated by Bain and Hurley (2011). Specifically, the population from north-western Laos is separated from that from northern Vietnam; both are found in the North-western Unit of Bain and Hurley (2011). In addition, populations in the central Annamites (Clade A) can be subdivided into at least two subclades. Within the central and southern Annamites subclade, three genetically-distinct populations were also identified. One is represented by sample SAC 15, which occurs north of Hai Van Pass, a well-known biogeographic boundary in the country. Another population denoted by sample SAC 26 is found in Khanh Hoa Province, southern Annamites and the remaining samples, SAC 27, SAC 28 and (12) occurring from Quang Nam to Binh Dinh Provinces (Table 1, Figs 2, 3). This subclade also exhibited the highest genetic divergence, around 1.8%, compared to other subclades in Vietnam and Laos (Table 3). Additional samples from this region will likely help to discover more diversity in this subclade.

Our new samples help to identify two novel subclades of *Sacalia quadriocellata*, one from northern Annamites, Nghe An Province, Vietnam and the other from north-eastern Laos, Houaphan Province, Lao PDR. Furthermore, two other subclades are more well-defined in terms of distribution with the addition of samples, SAC 11, 12 and 13 from this study. Previously, with only one known locality from sample (20), Shi et al. (2008) hypothesised that other samples of unknown locality, samples (17) to (24), share the same location. However, our analyses suggest that samples (20) to (24) belong to northern Vietnam's clade, whereas samples (17) to (19) originate from north-eastern Vietnam because they cluster with samples, SAC 11, 12 and 13 from Quang Ninh Province (Table 1, Figs 2, 3). The analyses also show that the subclade from central and southern Annamites, which was represented only by sample (12) from Quang Nam Province, is genetically much more diverse than previously determined. More samples from known localities in northern Vietnam, Hainan Island, mainland China and potentially eastern Lao PDR will likely reveal even higher diversity within the species. More surveys should therefore be conducted in the gap areas in China, Laos and Vietnam.

More detailed analyses can support assignment of unknown and/or misidentified samples to subclades with known origin. In addition to three samples, (17) to (19), from Shi et al. (2008), now revealed to be from north-eastern Vietnam, the identity of two samples from GenBank is also confirmed. One sample with accession number EF088646, which was misidentified as *Sacalia quadriocellata*, is, in fact, *S. bealei* as shown by our study. Another sample with accession number GU320209 belongs to northern Vietnam's subclade. Two samples, SAC 19 and 20, confiscated from Pleiku City, Gia Lai Province, were assigned to two different subclades, with SAC clustering

with other samples from central and southern Annamites and specifically most closely related to SAC 28 from Song Thanh Nature Reserve, Quang Nam Province and SAC 20 with those from central Annamites in Laos and Vietnam.

With the power to determine geographic provenance of unknown and misidentified samples, the application of phylogenetic and network analyses, based on informative barcoding genes, can help to track the origin of confiscated individuals from the trade. This is especially true when georeferenced samples are included, as shown in this and other studies (Welton et al. 2013; Siler et al. 2014; Zhang et al. 2015; Gaubert et al. 2016; Summerell et al. 2019; Kongrit et al. 2020; Ngo et al. 2020). Information of origin helps to identify hotspots of illegal hunting activities and provide better management strategies for conservation breeding programmes to avoid intermixing between animals from independently evolving lineages (Blair et al. 2017; Ngo et al. 2020). The population-level assignment can therefore assist monitoring of farmed animals to prevent laundering of wild turtles and other wildlife species (Ogden et al. 2009; Sigouin et al. 2017).

The key for proper assignment involves accurately georeferenced samples and selection of informative molecular markers, which can differentiate genetically-distinct populations (Ogden et al. 2009; Ogden and Linacre 2015; Blair et al. 2017). As turtles have declined drastically all over Asia (Horne et al. 2012; Rhodin et al. 2018), it is very challenging to sample animals from their native habitats. Historically, the Four-eyed Turtle had a low trade demand, but its market value has risen significantly, probably because natural populations of other turtle species have been extirpated (Tran et al. 2016). The recent trend has driven many turtle populations, including the Four-eyed turtles, in Vietnam to the brink of extinction (Tran et al. 2016; Pham et al. 2018). Our extensive field survey efforts in this study yielded only five wild-caught turtles. Due to this rarity, samples collected from the local trade, which are more readily available (Ly et al. 2011; McCormack et al. 2011; Pham et al. 2018), can substitute the field-collected ones in providing useful information on the local population. In this study, samples collected from the local trade helped to reveal at least one unknown subclade from north-eastern Laos. Others, i.e. SAC 11, 12 and 13, clarified the provenance of samples (17), (18) and (19) from Shi et al. (2008) or showed that populations from Thua Thien Hue Province (SAC 15) and Khanh Hoa Province (SAC 26), the southernmost locality of the Four-eyed Turtle's range, are genetically distinct from known populations.

These and other divergent populations clearly need more attention in future research to assess both their taxonomic and conservation status. Lin et al. (2018) suggested that the population from Hainan Island (samples 28–38 in Clade C of Fig. 3) represents a distinct species, but their morphological comparison included only specimens from Hainan and Guangdong and Guangxi and the results seemed to contradict the findings by Shi et al. (2008), specifically regarding the shape formed by the inner edges of pseudo-eyes on the back of the head and coloured patches in the lower jaw. The taxonomic revision is therefore unwarranted and the issue requires further investigation.

Population assignment can support releasing confiscated animals back to their natural range. Success of re-introduction programmes relies on a good understanding

of physiological demand of targeted species (IUCN/SSC 2013; Tarszisz et al. 2014). As an ectotherm, turtles are sensitive to changes of microclimate and cannot easily adapt if they are translocated to non-native habitat with different climate regimes (Butler 2019). In case of the Four-eyed Turtle, it would be detrimental to release animals originating from south-central Vietnam to northern Vietnam, because the two regions have very different climatic conditions (Sterling et al. 2006). In addition, the evolutionary differentiations between the populations, which have been likely facilitated by natural barriers of rivers and mountains, suggest they might have been isolated from each other for a long period of time with little evidence of gene flow. The release of animals with alien genotypes into a local population can lead to problems of outbreeding as they possess different adaptive potentials (Frankham et al. 2011; Weeks et al. 2011). The unwanted genetic pollution can result in extinction or compromise of local genetic pools (Butler 1994; Rhymer and Simberloff 1996; Fitzpatrick and Shaffer 2007). Genetic pollution through hybridisation between artificially-introduced and native turtles has been reported in several Asian countries (Fong and Chen 2010; Suzuki et al. 2014; Gong et al. 2018). Without sufficient guidance from genetic research, it would be almost impossible to reliably determine the provenance of four-eyed turtles confiscated from the trade, because only few morphological characters have been found to diagnose the populations (Shi et al. 2008).

The accelerated rate of wildlife trafficking has already complicated the issue of genetic pollution, especially in developing countries like Vietnam, where resources for keeping confiscated organisms are limited. Pressures to release the animals of unknown origin back to the wild can easily force errors in selecting appropriate sites. To date, the country has no specific regulation for translocation programmes and, in many cases, animals have been arbitrarily released to nearest sites of confiscated locations (Nguyen et al. 2017). However, this is often not where they come from, as our study clearly shows that two four-eyed turtles rescued from Pleiku have two different origins. In addition, the government has not provided any guidelines for rescuing and releasing confiscated animals back to the wild, which results in the re-introduction of unhealthy and non-native individuals (Nguyen et al. 2017). Government funding for combating wildlife trade is insufficient, let alone for genetic and disease screening. In our experience, resources for rescuing and releasing animals in accordance to the IUCN/SSC guidelines have largely come from international organisations and will likely to be the case in the foreseeable future.

Conclusion and recommendations

Pervasive international wildlife trade has resulted in a large volume of confiscated individuals with unknown origin. Consequently, there is a growing need for rescue centres and conservation breeding programmes around the world to take these animals into their facilities for rehabilitation and breeding. *Ex-situ* management should take into account the risks of mixing genetically-distinct lineages in captive facilities and

re-introducing non-native individuals to natural habitats. Detailed phylogeographic studies, employing field collected and local trade samples, can help to determine geographic provenance of confiscated animals and minimise impacts of the problems. Unfortunately, this kind of information is not available for many trade-targeted species, forcing managers to make difficult choices. To better manage the species, it is therefore a priority for conservation programmes to undertake phylogeographic works and genetically screen their captive colonies, especially when morphological characters to reliably diagnose geographically-distinct populations are lacking. To improve conservation of the Four-eyed Turtle in Vietnam, we recommend a genetic screening initiative to maintain genetic integrity of captive lineages. In addition, field and interview surveys should be conducted in gap areas in southern China, eastern Laos and northern Vietnam to clarify the genetic identity of the populations. Studies on population status and habitat suitability are also critically needed to establish areas for future releases of captive turtles. It is equally important that the government issues a set of criteria, including required standards for health and genetic profile of captive animals, to guide re-introduction activities. Without these comprehensive measures, biodiversity will be in great danger posed by genetic pollution from introduced non-native sources. Our research again underscores the IUCN's One Plan Approach, which aims to develop integrative strategies to combine *in situ* and *ex situ* measures with groups of experts for the purpose of species conservation.

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